Biochem. J. (1979) **184**, 619–626
Printed in Great Britain

Identification of a Defence Mechanism *in vivo* against the Leakage of Enterokinase into the Blood

By David A. W. GRANT,* Anthony I. MAGEE,* David MEEKS,† Christopher REGAN,† Douglas R. BAINBRIDGE† and John HERMON-TAYLOR*
**Department of Surgery, St. George's Hospital Medical School, Cranmer Terrace, London SW17 0RE, U.K., and †Clinical Immunology Laboratory, Department of Immunology, The London Hospital, Whitechapel, London E1 1BB, U.K.*

(*Received* 30 *April* 1979)

1. The serum proteinase inhibitors α_1-antitrypsin, α_2-macroglobulin, inter-α-trypsin inhibitor and C_1-esterase inhibitor were found not to affect the catalytic activity of human enterokinase, whereas bovine trypsin activity was modified essentially as expected. Enterokinase was also not inhibited by Trasylol (trypsin inhibitor from bovine lung) or bovine pancreatic trypsin inhibitor. No other component in human or mouse serum complexing with enterokinase was identified. 2. Human enterokinase administered intravenously into mice was rapidly cleared from the circulation with a half-life of 2.5 min. This removal was not the result of the difference in species, since partially purified mouse enterokinase was cleared at the same rate as the human enzyme. Clearance was mediated by recognition of the carbohydrate portion of enterokinase and not through specific recognition of its catalytic site. Immunofluorescent staining showed that the enzyme accumulated in the liver. Attempts to block the clearance by the simultaneous infusion of competing glycoproteins suggested that enterokinase was taken up by hepatocytes. Of the glycoproteins tested only two, human lactoferrin (terminal fucosyl $\alpha 1 \rightarrow 3$ *N*-acetylglucosamine) and bovine asialo-fetuin (terminal galactosyl $\beta 1 \rightarrow 4$ *N*-acetylglucosamine) were weakly competitive. Two inhibitors of endocytosis, Intralipid and Triton WR1339, failed to delay the removal of enterokinase. It is proposed that enterokinase is cleared from the circulation by an as yet uncharacterized hepatocyte receptor.

Intestinal protein digestion is largely dependent on the proteinases of pancreatic exocrine secretion, whose activation cascade is triggered in the limited proteolytic conversion of trypsinogen into trypsin by enterokinase. Immunofluorescence studies in man have demonstrated enterokinase on the brush border and glycocalyx of the enterocytes of the duodenum and upper 10–20 cm of jejunum (Hermon-Taylor *et al.*, 1977). Although the end point of proteolysis during digestion in the small intestine is the liberation of tripeptides, dipeptides and individual amino acids (Matthews & Adibi, 1976), there is evidence that some proteins, such as albumin (Warshaw *et al.*, 1974) and immunoglobulin G and ferritin (Williams & Hemmings, 1978), as well as dextrans (Tagesson *et al.*, 1978) may pass intact across normal intestinal epithelium to enter the circulation. There is also evidence that the absorption of native proteins may occur as a result of mucosal damage (Rhodes & Karnovsky, 1971; Worthington *et al.*, 1978). Morphological evidence of widespread mucosal damage to the upper small intestine is well documented in animals and man after acute ethanol ingestion (Rubin *et al.*, 1972; Baraona *et al.*, 1974). These changes affect the mucosa within the segment of greatest enterokinase activity, and it is probable that they are associated with the inappropriate absorption of the enzyme into the circulation. Such considerations may be relevant to the development of acute alcoholic pancreatitis, the pathogenesis of which is still incompletely understood (Hermon-Taylor, 1977).

The serum trypsin inhibitors form the principal defences against circulating active proteinases. Serine proteinases other than enterokinase, such as trypsin and thrombin, complex directly with or are transferred from other serum inhibitors to α_2-macroglobulin (Barrett & Starkey, 1973; Ohlsson *et al.*, 1971), and the complexes are then cleared from the circulation by the macrophages of the reticuloendothelial system (Ohlsson, 1971). It is not known whether enterokinase can be inhibited or eliminated by these mechanisms. Alternatively the heavily glycosylated enzyme may be cleared from the circulation by hepatocyte glycoprotein receptors (Ashwell & Morell, 1974). The present paper describes an investigation to determine whether enterokinase can persist in the circulation or if it is removed by either of these routes.

Materials and Methods

Pure human enterokinase was prepared by affinity chromatography as previously described (Grant *et al.*, 1978). Human α_2-macroglobulin (78% pure), α_1-antitrypsin (90% pure) and C_1-esterase inhibitor used in these studies were provided by the American Red Cross National Fractionation Centre, Bethesda, MD, U.S.A., with the partial support of the National Institutes of Health grant no. HL 13881. Inter-α-trypsin inhibitor was a gift from Dr. M. Steinbuch, Centre Nationale Transfusion Sanguine, Paris, France. Bovine pancreatic trypsin inhibitor, twice-crystallized bovine trypsin, once-crystallized bovine trypsinogen, bovine fetuin, human colostrom lactoferrin, diagnostic kit no. 281 for assaying leucine aminopeptidase and α-*N*-benzoyl-L-arginine ethyl ester hydrochloride (Bz-Arg-OEt) were from Sigma (London) Chemical Co., Poole, Dorset, U.K. Sephadex G-100, Sepharose 4B and gradient polyacrylamide gels PAA 4/30 were from Pharmacia (G.B.) Ltd., London W5 5SS, U.K. Trasylol was from Bayer (U.K.) Ltd., Haywards Heath, Surrey, U.K. Fucoidin and mannan were from Koch–Light Laboratories, Colnbrook, Bucks., U.K., and [^{3}H]-acetic anhydride was supplied by The Radiochemical Centre, Amersham, Bucks., U.K. Intralipid was from KabiVitrum, London W5 2TH, U.K., and Mulgofen BC-720 was from GAF (Great Britain), Colnbrook, Bucks., U.K. Glycyl-L-tetra-aspartyl-L-lysine β-naphthylamide was from Bachem Feinchemikalien A.G., CH-4416 Bubendorf, Switzerland. Purified blood-group-H substance (MH/PI/W5) was a gift from Professor W. Watkins, Clinical Research Centre, Harrow, Middx., U.K., and crude blood-group-A substance was a gift from Dr. T. Kristiansen, Institute of Biochemistry, Biomedicum, Uppsala, Sweden. Human carcinoembryonic antigen (COA/CD/4Aa) and Triton WR1339 were gifts from Dr. P. Thomas, Ludwig Institute, Royal Marsden Hospital, Sutton, Surrey, U.K. Asialo-fetuin was kindly supplied by Dr. J. Whately, Department of Biochemistry, St. George's Hospital Medical School. Human γ-globulin was prepared from fresh serum by precipitation in 40% (w/v) $(NH_4)_2SO_4$ and resolubilization in 10mM-sodium phosphate buffer, pH7.3, containing 150mM-NaCl. After dialysis against the same buffer the material was used without further purification. Aggregation of γ-globulin was achieved by warming at 63°C for 20min (Embling *et al.*, 1978).

Enzyme assays

Residual enterokinase and trypsin activities after incubation with purified serum proteinase inhibitors were determined by the methods of Baratti *et al.* (1973) and Schwert & Takenaka (1955) respectively. Trypsin was 50% active by weight, as determined by the methods of Hixson & Nishikawa (1973) and Chase & Shaw (1967). One unit of enterokinase activity was defined as that amount of enzyme producing 1nmol of active trypsin/min at 37°C. One unit of trypsin activity was defined by Schwert & Takenaka (1955) as that amount of enzyme producing a change of 0.001 A_{253} unit/min with Bz-Arg-OEt as substrate. Enterokinase activity in mouse serum was determined by the method of Grant & Hermon-Taylor (1979), with glycyl-L-tetra-aspartyl-L-lysine β-naphthylamide as substrate. One unit of activity was defined as that amount of enzyme hydrolysing 1pmol of substrate/h at 37°C.

Enzyme-inhibition studies

All assays were performed at 30°C in 36mM-Tris/HCl/14mM-Tris base, pH7.7, containing 0.1M-NaCl and 0.05M-$CaCl_2$ (buffer A). The molar concentration of each inhibitor was adjusted to allow for inactive material. Gel chromatography was performed at 4°C in 44mM-Tris/HCl/6mM-Tris base, pH7.7 (buffer B), and polyacrylamide-gradient-gel electrophoresis was carried out at room temperature in 50mM-glycine/NaOH buffer, pH9.8, containing 4mM-sodium dodecyl sulphate. Samples for electrophoresis were prepared by mixing with 0.2vol. of stock solution containing 0.6M-sodium dodecyl sulphate, 0.06M-dithiothreitol, 12% (w/v) sucrose, 3mM-EDTA and 0.003% Bromophenol Blue. Bovine serum albumin (mol.wt. 66000), β-galactosidase (mol.wt. 132000) and catalase (mol.wt. 232000) were used as molecular-weight standards. The concentrations of α_2-macroglobulin, C_1-esterase inhibitor and inter-α-trypsin inhibitor were quantified by using radial immunodiffusion plates (Hoechst Pharmaceuticals, Hounslow, Middx., U.K.). Protein concentrations were measured by the method of Lowry *et al.* (1951) with bovine serum albumin as the standard.

A 1–5-fold molar excess of either α_2-macroglobulin or α_1-antitrypsin was incubated for 15min and 30min respectively with either 100 units of trypsin (specific activity 12000 units/mg) or 0.05 unit of human enterokinase (specific activity 3950 units/mg). In addition 750 units of trypsin and 8.6 units of enterokinase were incubated with a 2-fold and 10-fold molar excess respectively of α_2-macroglobulin in a final volume of 0.5ml before application to a column (21cm × 1.5cm) of Sepharose 4B equilibrated in buffer B. Fractions (2.1ml) were collected and assayed for both enzymic and inhibitory activities.

A 1–6-fold molar excess of either C_1-esterase inhibitor or inter-α-trypsin inhibitor was incubated for 15min with either 100 units of trypsin or 0.05 unit of enterokinase. The experiments were repeated with a 1–4-fold molar excess of either Trasylol or bovine trypsin inhibitor with 100 units of trypsin, or

a 1–10-fold molar excess of the same inhibitors with 0.05 unit of enterokinase.

Finally 26 units of enterokinase was preincubated with 0.5ml of pooled whole human serum from healthy blood-group-AB subjects before chromatography on Sepharose 4B as described above. A similar study was carried out with fresh mouse serum. Fractions (1.3ml) were assayed for enzymic activity.

In all cases the residual esterolytic activity of trypsin against Bz-Arg-OEt and the ability of enterokinase/inhibitor mixtures to activate trypsinogen was determined.

Preparations of glycoproteins for simultaneous infusion with enterokinase

Unless otherwise indicated the glycoproteins tested were used without further purification.

Human blood-group-A substance was purified from a freeze-dried product of ovarian-cyst ascites fluid by the method of Kristiansen *et al.* (1969). Asialoagalacto-fetuin was prepared from commercially available fetuin by passing 200mg of material through a column (6cm×1.5cm) of wheat-germ lectin immobilized on Sepharose 4B equilibrated in 10mM-sodium phosphate buffer, pH7.2, containing 150mM-NaCl (Magee *et al.*, 1977); 7mg of asialoagalacto-fetuin was desorbed by the addition of 50mM-*N*-acetyl-D-glucosamine to the buffer.

Preparation of mouse enterokinase

For this, 200 female CBA mice (approx. 25g each) were killed and the upper small intestine was removed. The tissue was cut lengthwise to expose the mucosal surface and finely chopped into 500ml of ice-cold 10mM-glycine/NaOH buffer, pH9.0, containing 0.5% (w/v) Mulgofen BC-720 (buffer C). The material was homogenized for 15s in an MSE Atomixer and then left on ice for 1h. After being centrifuged at 1000*g* for 15min the supernatant was retained and the gelatinous residue re-extracted with a further 350ml of buffer C.

The supernatants were combined and mixed with approx. 250ml of DEAE-cellulose equilibrated in 10mM-sodium phosphate buffer, pH7.6 (buffer D). After being stirred for 30min the mixture was centrifuged at 1000*g* for 15min and the supernatant which contained approx. 80% of the total enterokinase activity, was retained. The remaining 20% was recovered by washing the DEAE-cellulose with an equal volume of buffer D containing 0.3M-NaCl. The two pools were combined and dialysed against 10 litres of buffer D. The enzyme was then chromatographed on a column (30cm×2.54cm diam.) of DEAE-cellulose. Elution was by a linear gradient of 0–400mM-NaCl from two 250ml reservoirs. Fractions (10ml) containing enterokinase were pooled and dialysed against 34mM-Tris/HCl/16mM-Tris base, pH8.4 at 4°C. Purification was then continued as described for the human enzyme (Grant *et al.*, 1978). The final preparation was free of intestinal aminopeptidase and naphthylamidases.

Radiolabelling of enterokinase

Approx. 7.5mCi of [^{3}H]acetic anhydride solubilized in 0.5ml of acetonitrile was quickly mixed with 2ml of 0.05M-Tris/HCl, pH8.4, containing 38 units of human enterokinase (9.0μg of enzyme protein) and briefly agitated, and then left for 1h at 25°C. The mixtures was dialysed for 36h against 3×2.5 litres of ice-cold water. The pool was divided into two equal parts. One part was made up to 5ml with 20mM-sodium acetate buffer, pH6. Then 50μl of 1M-$NaIO_4$ was rapidly added and the solution left on ice for 7h. The reaction was quenched by the addition of 0.6ml of 1M-ethylene glycol and by subsequent dialysis against 10mM-sodium phosphate buffer, pH7.3, containing 0.15M-NaCl.

For the second fraction, catalytically active labelled enzyme was separated from material that had been denatured, by using affinity chromatography on a 10ml bed of immobilized glycylglycyl-*p*-aminobenzamidine–Sepharose 4B (Grant *et al.*, 1978). Denatured enzyme (10ml) that did not bind to the column was retained for use in the clearance studies. For the active enzyme eluted from the column the radioactivity (c.p.m.) per unit was determined before dividing the pool (8.8ml) into two equal portions, one of which was dialysed against 0.9% NaCl. The other portion was dialysed against 10mM-sodium phosphate buffer, pH7.2, containing 0.15M-NaCl, before the addition of 2mg of Tos-Lys-CH_2Cl (7-amino-1-chloro-3-L-tosylamidoheptan-2-one; TLCK) (Maroux *et al.*, 1971). After 6h at 25°C the inactivated enzyme was dialysed extensively against 0.15M-NaCl. The samples of active and of inactivated enzyme were made up to 7.25ml with 0.9% NaCl and the radioactivity (c.p.m.) per unit was redetermined before intravenous administration into the mice.

Clearance of enterokinase from mouse serum

For this, 7–8-week-old female CBA mice weighing 16–20g were used throughout the study and were not starved before experiments. Iso-osmotic 0.9% NaCl (0.5ml) containing 0.65 unit of enterokinase alone, or enterokinase plus the material under test, or modified ^{3}H-labelled enterokinase, was injected into the tail vein and duplicate animals were killed after 1, 2, 5, 10, 20 and 40min. Blood was collected under ether anaesthetic by severing the brachial vein. Serum enterokinase activity was measured (Grant & Hermon-Taylor, 1979) or 0.1ml samples of serum were counted for radioactivity in 5ml of scintillant stock solution, made up from 1 litre of toluene, 50ml of Triton X-100, 5.5g of 2,5-diphenyloxazole and 0.05g of 1,4-bis-(5-phenyloxazol-2-yl)benzene

by using a SL30 Intertechnique liquid-scintillation counter.

For experiments with Intralipid and Triton WR1339 the mice received four prior intravenous injections at 24h intervals of either 0.5ml of Intralipid or 0.25ml of 10% (w/v) Triton WR1339 in iso-osmotic 0.9% NaCl. Clearance studies were performed on day 5.

Immunofluorescence studies

The liver, spleen and kidneys were removed from mice 20min after the injection of enterokinase. Tissues were snap-frozen in isopentane/liquid N_2, and 5μm cryostat sections were stained by the indirect immunofluorescent technique. Specific rabbit anti-(human enterokinase) serum (Hermon-Taylor *et al.*, 1977) (diluted 1:10) was applied to the sections; after 30min at room temperature the sections were washed in 10mM-sodium phosphate buffer, pH7.3, containing 0.15M-NaCl, and fluorescein isothiocyanate–sheep anti-(rabbit immunoglobulin) conjugate (Wellcome Reagents, Beckenham, Kent, U.K.) was applied for 30min. Sections were mounted in glycerol and examined by u.v. epi-illumination. Normal rabbit serum and conjugate controls were also run.

Results

Inhibition studies

Trypsin activity against the low-molecular-weight synthetic substrate Bz-Arg-OEt was completely inhibited by a 4.5-fold molar excess of the preparation of α_1-antitrypsin, a 3-fold molar excess of the preparation of inter-α-trypsin inhibitor, a 2-fold molar excess of Trasylol and by an equimolar amount of bovine pancreatic trypsin inhibitor. A 5-fold molar excess of α_2-macroglobulin did not inhibit this esterolytic activity of trypsin. However, two peaks of activity were found after Sepharose 4B chromatography corresponding to the elution positions of α_2-macroglobulin/trypsin complex and free trypsin, indicating that the expected active complex between the enzyme and inhibitor had been formed (Barrett & Starkey, 1973). In addition the 85000-mol.wt. subunits of α_2-macroglobulin, formed after binding trypsin, were identified by gradient sodium dodecyl sulphate/polyacrylamide-gel electrophoresis (Harpel, 1973).

The activation of trypsinogen by enterokinase was not inhibited at any molar concentration of these biological inhibitors. Unlike trypsin, the formation of a complex between α_2-macroglobulin and enterokinase could not be identified by Sepharose 4B chromatography or sodium dodecyl sulphate/polyacrylamide-gel electrophoresis. In addition, enterokinase did not complex with any component present in blood-group-AB serum, since all the enzyme activity was eluted as a symmetrical peak at its normal position after Sepharose 4B chromatography.

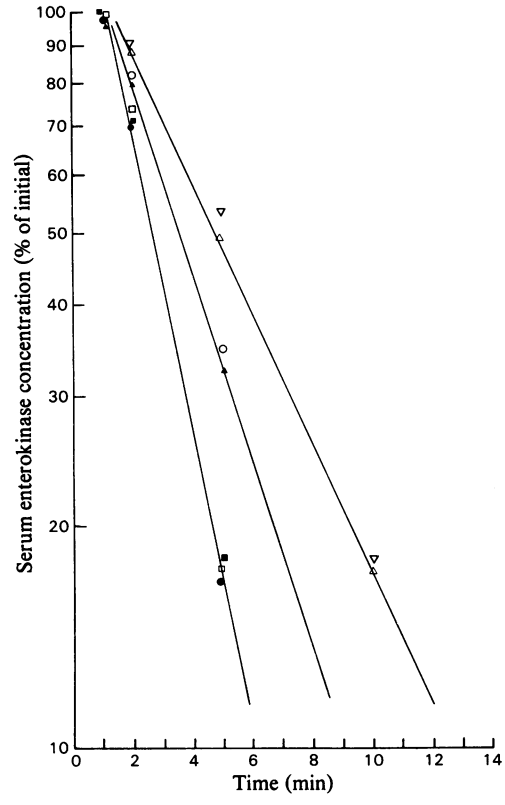

Fig. 1. *Effect of simultaneous intravenous injections of competing glycoproteins on the clearance of enterokinase from the circulation of the mouse*

Enterokinase alone (□); approx. 2×10^4-fold molar excess of aggregated human immunoglobulin G (●), bovine asialoagalacto-fetuin (■), human lactoferrin (▲) and bovine asialo-fetuin (○); approx. 6×10^4-fold molar excess of human lactoferrin (△); 2mg of fucoidin (▽). For details see the text.

Clearance studies

The circulatory half-life of human enterokinase after intravenous infusion into mice was 2.5min (Fig. 1). The effects of putative competitors on this clearance time are listed in Table 1. In no case was the half-life prolonged beyond 5min and only two of the seven glycoproteins tested competed with enterokinase for clearance (Fig. 1). The prior infusion of either Intralipid or Triton WR1339 had no effect on the half-life. Radio-labelled human enterokinase was cleared from the circulation at the same rate irrespective of whether it was catalytically active or had

Table 1. *Effect of competing glycoproteins on the uptake of circulating enterokinase*
For purified human enzyme, 0.52 unit (125ng of enzyme protein) was used throughout the study; for mouse enzyme the number of units was equivalent to the human enzyme. For full details see the text.

Injected sample	$t_{\frac{1}{2}}$ (min)
Purified human enterokinase	2.5
+2mg of yeast mannan	2.5
+0.075mg of human carcinoembryonic antigen	2.5
+0.5mg of bovine asialoagalacto-fetuin	2.5
+1.5mg of aggregated human γ-globulin	2.5
+2mg of human blood-group-H substance	2.5
+2mg of human blood-group-A substance	2.5
+0.5mg of bovine asialo-fetuin	4.0
+1.35mg of human lactoferrin	4.0
+3.5mg of human lactoferrin	5.0
+2mg of fucoidin	5.0
Partially purified murine enterokinase	2.5

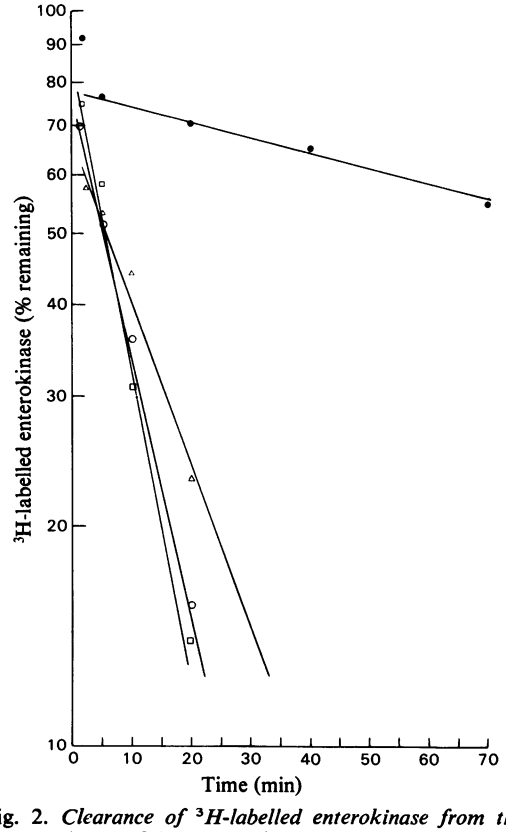

Fig. 2. *Clearance of ^{3}H-labelled enterokinase from the circulation of the mouse after intravenous injection*
□, Catalytically active enzyme; ○, Tos-Lys-CH_2Cl-inactivated enzyme; △, denatured inactive enzyme; ●, periodate-oxidized inactive enzyme. For details see the text.

been inactivated by denaturation or by active-site titration by using Tos-Lys-CH_2Cl. In all three cases the half-life was approx. 5min (Fig. 2). Periodate oxidation of the radiolabelled enzyme before infusion greatly prolonged its survival in serum, and over half the injected material could still be detected after 70min. Mouse enterokinase showed the same clearance kinetics as the human enzyme. Immunofluorescent staining of liver sections demonstrated enterokinase at the biliary canalicular membrane 20min after intravenous injection; Kupffer-cell staining was also seen. Sections of spleen showed some fluorescence in peripheral sinusoid cells, but kidney was consistently negative. Normal rabbit serum, taken before immunization with human enterokinase, and conjugated sheep anti-(rabbit immunoglobulin) controls were negative on all tissues.

Discussion

Both enterokinase and trypsin are serine proteinases and share trypsinogen as a common substrate. Although all the inhibitors tested bound trypsin essentially as described by other workers (Figarella *et al.*, 1975), enterokinase interacted with none of them. The interaction of trypsin with α_2-macroglobulin involves cleavage of a susceptible bond in the inhibitor, with a resultant conformational change that may entrap the proteinase within the macromolecule (Barrett & Starkey, 1973); the failure of enterokinase to complex with this inhibitor suggested either that a susceptible bond was not available or that the interaction between enterokinase (mol.wt. 300000) and α_2-macroglobulin (mol.wt. 720000) was sterically unfavourable. The largest proteinase known to complex with this serum inhibitor is plasmin

(mol.wt. 90000) (Barrett & Starkey, 1973). Since human enterokinase has been shown to exhibit blood-group-A-like immunoreactivity in rabbits (Hermon-Taylor *et al.*, 1977), it was important to exclude immune-complex-formation as a mechanism of its clearance from the circulation. No enterokinase-containing complex was identified with human or mouse serum. Taken together these findings indicate that serum components do not inactivate, or bind to, the enzyme and that enterokinase is capable of circulating in its catalytically active form. Since immunoreactive trypsinogen has been identified in human serum (Largman *et al.*, 1978) and there is evidence to support the concept of an entero-pancreatic recirculation of zymogen (Rothman, 1978), there would appear to be a need for a defence mechanism for the rapid elimination of enterokinase that may be sequestered into the blood.

The clearance of intravenously injected enterokinase in this study was found to be similar to that described for the removal of other human glycoproteins such as carcinoembryonic antigen (Thomas & Jones, 1978), lymphoblastoid interferon (Bose & Hickman, 1977) and lactoferrin (Prieels *et al.*, 1978). Clearance was rapid, with a serum half-life of only 2.5 min and was not due to active-site recognition. The rate of removal of radiolabelled active enterokinase was the same as for the removal of labelled inactive enterokinase. The method of inactivation was not critical; material that had been denatured during isotopic labelling or enzyme that had been treated with Tos-Lys-CH_2Cl (Maroux *et al.*, 1971) behaved identically. The circulating half-life of 5 min for ^3H-labelled enterokinase was double that found by monitoring catalytic activity. This would suggest that there was some loss of the acetyl label from the enzyme *in vivo*, not associated with enzyme activity, but which prolonged the detection of ^3H in serum. This illustrates the advantage of following the enzyme clearance with a specific substrate as a monitor of behaviour *in vivo*.

It was possible that the rapid clearance of human enterokinase in the mouse was a species artefact. This consideration is also applicable to other studies in which glycoproteins purified from one species have been infused into another. In order to check this, a small quantity of mouse enterokinase was partly purified by affinity chromatography. The material was free of intestinal aminopeptidase and naphthylamidases, indicating that any hydrolysis of the artificial substrate glycyl-L-tetra-aspartyl-L-lysine β-naphthylamide in the assay was due to enterokinase alone. That the clearance was not species-specific was suggested by the finding that an equal number of units of mouse enterokinase were cleared at a rate identical with that for the human enzyme.

Periodate oxidation of the radiolabelled enzyme before injection blocked the rapid clearance of enterokinase from the serum, extending the half-life beyond 1 h. This suggested that clearance was dependent on the recognition of an intact carbohydrate moiety. Hepatocyte receptors have been described for glycoproteins containing terminal galactose-linked β-1,4 to *N*-acetylglucosamine (Morell *et al.*, 1971) and terminal fucose linked α-1,3 to *N*-acetylglucosamine (Prieels *et al.*, 1978), whereas carbohydrate side chains terminating in mannose (Brown *et al.*, 1978; Schlesinger *et al.*, 1978) or *N*-acetylglucosamine (Stockert *et al.*, 1976) are recognized by Kupffer-cell surface receptors. Human enterokinase is a heavily glycosylated enzyme containing 57% carbohydrate by weight (Grant & Hermon-Taylor, 1976). Immunofluorescent staining of liver, spleen and kidney showed the enzyme to be located at the biliary canalicular membrane 20 min after injection, although staining was also seen in Kupffer cells and in the sinusoidal cells in the spleen. The specificity of anti-(human enterokinase) for the active-site region of the enzyme has been established, and cross-reaction with human blood-group-A substance eliminated (Hermon-Taylor *et al.*, 1977). Earlier studies on sections taken 1 h after intravenous administration of enzyme had suggested that enterokinase was cleared by the Kupffer cells and subsequently transferred to hepatocytes (Grant *et al.*, 1979). However, Thomas *et al.* (1977) have demonstrated a time-dependent transfer of native human carcinoembryonic antigen from Kupffer cells to hepatocytes and further experiments were performed in which mice were killed 20 min after intravenous administration. In this case the enzyme was mainly located in the hepatocytes. Further experiments showed that clearance of enterokinase from the serum was unaffected by large doses of Intralipid or Triton WR1339, which are known to interfere with endocytosis (Cotmore & Carter, 1973; Wisse, 1977), and this suggested that the enzyme was preferentially cleared by hepatocytes, although some may be transferred to, or directly taken up by, Kupffer and other sinusoidal cells. This is in agreement with the work of Thomas *et al.* (1977), who showed that both asialo-α_1-acid glycoprotein and asialo-carcinoembryonic antigen (both containing terminal galactose linked β-1,4 to *N*-acetylglucosamine) were primarily located in hepatocytes 15 min after intravenous injection, although some Kupffer-cell uptake could also be observed.

Of the glycoproteins tested in competition, only the simultaneous infusion of about 2×10^4-fold molar excess of either bovine asialo-fetuin (terminal galactose $\beta 1 \rightarrow 4$ *N*-acetylglucosamine) or human lactoferrin (terminal fucose $\alpha 1 \rightarrow 3$ *N*-acetylglucosamine) prolonged the half-life of enterokinase, in both cases to 4 min. Both asialo-fetuin and lactoferrin have been shown to be hepatocyte directed (Ashwell & Morell, 1974; Prieels *et al.*, 1978).

Although the simultaneous infusion of 2mg of fucoidin affected enterokinase clearance, this may have been due to a heparin-like activity. Thrombin clotting-time assays showed that 2mg of fucoidin had an activity equivalent to 0.25mg of heparin. Berghem *et al.* (1977) demonstrated that heparin impairs the hepatic clearance of aggregated albumin and proposed that this was due to an increase in the negative charge density at the cell surface, impairing the attachment of negatively charged macromolecules. This may have applied to human enterokinase, which is an acidic glycoprotein, pI 4.78 (Grant & Hermon-Taylor, 1976).

Most of the glycoproteins tested failed to affect the clearance of enterokinase. Native human carcinoembryonic antigen (approx. 700-fold molar excess over enterokinase) and aggregated human immunoglobulin (approx. 2×10^4-fold molar excess) have been shown to be bound preferentially by Kupffer cells (Thomas & Jones, 1978; Embling *et al.*, 1978). Asialoagalacto-fetuin (2.2×10^4-fold molar excess) contained terminal *N*-acetylglucosamine residues, since it was isolated by lectin-specific affinity chromatography, and glycoproteins containing this determinant have also been shown to be taken up by phagocytic cells (Stahl *et al.*, 1978). Both human blood-group-active substances A and H failed to inhibit enterokinase clearance when infused at about 2×10^4-fold molar excess. Human blood-group-H substance contains terminal fucose residues linked α-1,2 to galactose, and this is the precursor for blood-group-A substances, which has an additional *N*-acetylgalactosamine residue linked α-1,3 to the galactose residue (Watkins, 1966).

Since the only competing glycoproteins that delayed enterokinase clearance were those known to be hepatocyte-directed, these results support the immunofluorescence evidence that circulating active enterokinase may be cleared by the hepatocyte. The mechanism is clearly dependent on an intact carbohydrate moiety, but the half-life of serum enterokinase was prolonged to only 4min in the presence of approx. 10^4-fold molar excess of competing material. This was either because the clearance system was not fully saturated even at this concentration of competing material, or, more likely, that the terminal fucose and galactose residues were only weakly competitive and that enterokinase had a low affinity for the two established hepatocyte oligosaccharide receptors; a 3-fold increase in the lactoferrin load only extended the half-life of the enzyme by 1min more. This would suggest that the clearance of enterokinase was dependent on recognition by a third type of hepatocyte receptor with a different preferred carbohydrate configuration. That such a configuration on enterokinase involves terminal fucose or galactose residues is supported by the persistence of the enzyme in the circulation after periodate oxidation of these sugars. Fucose and galactose may comprise approx. 30% of the enzyme by weight (Grant & Hermon-Taylor, 1976). Of the fucose-terminal glycoproteins only lactoferrin with fucose $\alpha1\rightarrow3$ *N*-acetylglucosamine was weakly competitive, whereas fucose $\alpha1\rightarrow6$ *N*-acetylglucosamine (human immunoglobulin G) (Kornfeld & Kornfeld, 1976) and fucose $\alpha1\rightarrow2$ galactose (blood-group-H substance) (Watkins, 1966) had no effect. The specificity of the fucose linkages in recognition is supported by the finding of Prieels *et al.* (1978) that fucose $\alpha1\rightarrow2$ galactose (asialo-mucin) failed to inhibit human lactoferrin clearance by rat liver. The only terminal galactose configuration to be weakly competitive for enterokinase was galactose $\beta1\rightarrow4$ *N*-acetylglucosamine (asialo-fetuin). These considerations suggest that either or both fucose $\alpha1\rightarrow3$ *N*-acetylglucosamine and galactose $\beta1\rightarrow4$ *N*-acetylglucosamine contribute to the recognized carbohydrate conformation on enterokinase.

This work was supported in part by a grant from the Medical Research Council. We thank Dr. M. Steinbuch for her generous gift of inter-α-trypsin inhibitor.

References

Ashwell, G. & Morell, A. G. (1974) *Adv. Enzymol. Relat. Areas Mol. Biol.* **41**, 99–128

Baraona, E., Pirola, R. C. & Lieber, C. S. (1974) *Gastroenterology* **66**, 226–234

Baratti, J., Maroux, S., Louvard, D. & Desnuelle, P. (1973) *Biochim. Biophys. Acta* **315**, 147–161

Barrett, A. J. & Starkey, P. (1973) *Biochem. J.* **133**, 709–724

Berghem, L., Ahlgren, T. & Lahnborg, G. (1977) in *Kupffer Cells and Other Liver Sinusoidal Cells* (Wisse, E. & Knook, D. L., eds.), pp. 379–386, Elsevier/North-Holland, Amsterdam

Bose, S. & Hickman, J. (1977) *J. Biol. Chem.* **252**, 8336–8337

Brown, T. L., Henderson, L. A., Thorpe, S. R. & Baynes, J. W. (1978) *Arch. Biochem. Biophys.* **188**, 418–428

Chase, T. & Shaw, E. (1967) *Biochem. Biophys. Res. Commun.* **29**, 508–514

Cotmore, S. F. & Carter, R. L. (1973) *Int. J. Cancer* **11**, 725–738

Embling, P. H., Evans, H., Guttierez, C., Holborow, E. J., Johns, P., Johnson, P. M., Papamichail, M. & Stanworth, D. R. (1978) *Immunology* **34**, 781–786

Figarella, C., Negri, G. A. & Guy, O. (1975) *Eur. J. Biochem.* **53**, 457–463

Grant, D. A. W. & Hermon-Taylor, J. (1976) *Biochem. J.* **155**, 243–254

Grant, D. A. W. & Hermon-Taylor, J. (1979) *Biochim. Biophys. Acta* **567**, 207–215

Grant, D. A. W., Magee, A. I. & Hermon-Taylor, J. (1978) *Eur. J. Biochem.* **88**, 183–189

Grant, D. A. W., Magee, A. I., Perrin, J., Meeks, D., Bainbridge, D. & Hermon-Taylor, J. (1979) *FEBS Spec. Meet. Enzymes, abstr.* S3–75

Harpel, P. (1973) *J. Exp. Med.* **138**, 508–521
Hermon-Taylor, J. (1977) *Br. J. Hosp. Med.* **18**, 546–552
Hermon-Taylor, J., Perrin, J., Grant, D. A. W., Appleyard, A., Bubel, M. & Magee, A. I. (1977) *Gut* **18**, 259–265
Hixson, H. F. & Nishikawa, A. H. (1973) *Arch. Biochem. Biophys.* **154**, 501–509
Kornfeld, R. & Kornfeld, S. (1976) *Annu. Rev. Biochem.* **45**, 217–237
Kristiansen, T., Sundberg, L. & Porath, J. (1969) *Biochim. Biophys. Acta* **184**, 93–98
Largman, C., Brodrick, J. W., Geokas, M. C. & Johnson, J. H. (1978) *Biochim. Biophys. Acta* **543**, 450–454
Lowry, O. H., Rosebrough, N. J., Farr, A. L. & Randall, R. J. (1951) *J. Biol. Chem.* **193**, 265–275
Magee, A. I., Grant, D. A. W. & Hermon-Taylor, J. (1977) in *Affinity Chromatography: Proc. Int. Symp.* (Hoffman-Ostenhof, O., Breitenbach, M., Koller, F., Kraft, D. & Scheiner, O., eds.) pp. 287–290, Pergamon Press
Maroux, S., Baratti, J. & Desnuelle, P. (1971) *J. Biol. Chem.* **246**, 5031–5039
Matthews, D. H. & Adibi, S. A. (1976) *Gastroenterology* **71**, 151–161
Morell, A., Gregoriadis, G., Scheinberg, I. H., Hickman, J. & Ashwell, G. (1971) *J. Biol. Chem.* **246**, 1461–1467
Ohlsson, K. (1971) *Acta Physiol. Scand.* **8**, 269–272
Ohlsson, K., Ganrot, P. O. & Laurell, C. B. (1971) *Acta Chir. Scand.* **137**, 113–121
Prieels, J.-P., Pizzo, S., Glasgow, L. R., Paulson, J. C. & Hill, R. L. (1978) *Proc. Natl. Acad. Sci. U.S.A.* **75**, 2215–2219
Rhodes, R. S. & Karnovsky, J. J. (1971) *Lab. Invest.* **25**, 220–229
Rothman, S. S. (1978) *Biochim. Biophys. Acta* **509**, 374–383
Rubin, E., Rybak, B. J., Lindenbaum, J., Gerson, C. D., Walker, G. & Lieber, C. S. (1972) *Gastroenterology* **63**, 801–814
Schlesinger, P. H., Doebber, T. W., Mandell, B. F., White, R., DeSchryver, C., Rodman, J. S., Miller, M. J. & Stahl, P. (1978) *Biochem. J.* **176**, 103–109
Schwert, G. W. & Takenaka, Y. (1955) *Biochim. Biophys. Acta* **16**, 570–575
Stahl, P. D., Rodman, J. S., Miller, M. J. & Schlesinger, P. H. (1978) *Proc. Natl. Acad. Sci. U.S.A.* **75**, 1399–1403
Stockert, R. J., Morell, A. G. & Scheinberg, I. H. (1976) *Biochem. Biophys. Res. Commun.* **68**, 988–993
Tagesson, C., Sjodahl, R. & Thoren, B. (1978) *Scand. J. Gastroenterol.* **13**, 519–524
Thomas, P. & Jones, M. (1978) *Biochem. J.* **173**, 981–983
Thomas, P., Birbeck, M. S. C. & Cartwright, P. (1977) *Biochem. Soc. Trans.* **5**, 312–313
Warshaw, A. L., Walker, W. A. & Isselbacher, K. J. (1974) *Gastroenterology* **66**, 987–992
Watkins, W. (1966) *Science* **152**, 172–181
Williams, E. W. & Hemmings, W. A. (1978) *Proc. R. Soc. London Ser. B* **203**, 177–189
Wisse, E. (1977) in *Kupffer Cells and Other Liver Sinusoidal Cells* (Wisse, E. & Knook, D. L., eds.), pp. 33–60, Elsevier/North-Holland, Amsterdam
Worthington, B. S., Meserole, L. & Syrotuck, J. A. (1978) *Am. J. Dig. Dis.* **23**, 23–32